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Photoaffinity Labeling of the Sigma-1 Receptor with *N*-[3-(4-Nitrophenyl)propyl]-*N*-dodecylamine: Evidence of Receptor Dimers

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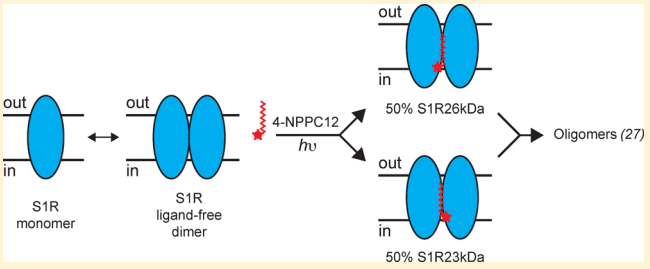
Supporting Information

ABSTRACT: The sigma-1 receptor is a ligand-regulated endoplasmic reticulum (ER) resident chaperone involved in the maintenance of cellular homeostasis. Coupling of the sigma-1 receptor with various ER and/or plasma membrane ion channels is associated with its ability to regulate the locomotor activity and cellular proliferation produced in response to sigma-1 receptor ligands. A number of endogenous small molecules bind to the sigma-1 receptor and have been shown to regulate its activity; these include progesterone, *N*,*N*-dimethyltryptamine, D-*erythro*-sphingosine, and/or other endogenous lipids. We previously reported the synthesis of long chain *N*-alkylamine derivatives and the characterization of the structure−activity relationship between the chain length of *N*-alkylamine and affinities at the sigma-1 receptor. Here, we present data demonstrating the photoincorporation of one of these *N*-alkylamine derivatives, *N*-[3-(4-nitrophenyl)propyl]-*N*-dodecylamine (4-NPPC12), to the sigma-1 receptor. Matrix-assisted laser desorption ionization time-of-flight and tandem mass spectrometry showed that 4-NPPC12 photoinserted at histidine 154 of the derivatized population of the sigma-1 receptor. Interestingly, light-dependent photoinsertion of 4-NPPC12 resulted in an enhanced electrophoretic mobility of only 50% of the derivatized receptor molecules as assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The proposed binding and reactivity of 4-NPPC12 evoke a ligand binding model for the sigma-1 receptor that likely involves a receptor dimer and/or oligomer.



The sigma-1 receptor belongs to a unique family of membrane receptors of which there are at least two subtypes, the sigma-1 (26 kDa) and sigma-2 (18 kDa) receptors. The sigma-1 and sigma-2 receptors can be distinguished by their pharmacological profiles, selectivities, functions, subcellular locations, and molecular masses. Because its sequence is available,[1] the sigma-1 receptor has been extensively evaluated and shown to possess chaperone activity.[2] The 18 kDa sigma-2 receptor has not yet been cloned, but recently, a 26 kDa membrane-localized progesterone receptor membrane component 1 (PCRMC1) has been shown to possess a sigma-2-like binding site.[3] Multiple lines of evidence indicate that the sigma-1 receptor plays an important role in the maintenance of cellular membrane potential via a direct and/or indirect modulation of various ion channels. These studies demonstrated that the sigma-1 receptor can regulate the function of voltage-gated potassium (K^+),[4] sodium (Na^+),[5] calcium (Ca^{2+}),[6] and chloride (Cl^-) channels[7] and expression of the human hERG potassium channel.[8] Direct physical interactions between the sigma-1 receptor and voltage-gated potassium channels[4] and between the sigma-1 receptor and the acid-sensing ion channel ASIC1a[9] have also been demonstrated, leading to the suggestion that the sigma-1 receptor may be a regulatory subunit for certain ion channels. Additionally, through its chaperone activity, the sigma-1 receptor can indirectly modulate entry of Ca^{2+} from the ER into the mitochondria via the stabilization of inositol 1,4,5-trisphophate receptor type 3 (IP_3R-3).[2,10]

Interest in the sigma-1 receptor arises because of its "promiscuous" interaction with a wide range of synthetic pharmacological compounds such as the antipsychotic haloperidol, the Ca^{2+} channel antagonist verapamil, the antidepressant fluvoxamine, and the central nervous system stimulant methamphetamine (for a literature review of sigma-1 receptor ligands see Su et al.[11] and of the sigma-1 receptor pharmacophore see Glennon et al.,[12] publications by Fontanilla et al.,[9] and Chu et al.[13]). Endogenously, the sigma-1 receptor has been suggested to bind a number of compounds, including the neurosteroid progesterone,[14] the hallucinogen *N*,*N*-dimethyltryptamine (DMT),[15] and/or sphingolipids such as D-*erythro*-sphingosine[16] and ceramides.[17] Binding of ligand to the sigma-1 receptor has the following cellular signaling consequences: modulation of the interaction between an ER resident chaperone GRP78/BiP and sigma-1 receptor,[2] regulation of the association of ankyrin B, IP_3 receptor and sigma-1 receptor tricomplex,[18] influencing the ion flux through different ion channels,[4−8] redistribution of

Received: November 9, 2012
Revised: January 16, 2013
Published: January 16, 2013

the sigma-1 receptor to different cellular locales,[17,19,20] and a reduction in the level of cellular oxidative stress.[21−23]

The sigma-1 receptor cloned from various species encodes a protein of 223 amino acids[1,24,25] that migrates on a sodium dodecyl sulfate (SDS)−polyacrylamide gel at 26 kDa. We have been involved in defining the ligand-binding region of the sigma-1 receptor using photoaffinity labels.[26−28] For example, we showed previously that a high-affinity sigma-1 selective photolabel [^{125}I]iodoazidococaine ([^{125}I]IACoc) photolabeled aspartate 188 located on steroid binding domain-like (SBDL) II,[26,29] [^{125}I]iodoazidofenpropimorph ([^{125}I]IAF) photolabeled both SBDLI and SBDLII,[27] and a radioiodinated benzophenone bifunctional cross-linking reagent tethered together SBDLI and SBDLII indicating an approximate 8 Å distance between these two domains.[30] Additionally, C-terminal truncations of the last 15 amino acids from the C-terminal region of the sigma-1 receptor showed reduced levels of [^{125}I]IACoc photolabeling.[26] A number of biochemical studies from our laboratories as well as others using site-directed mutagenesis have also demonstrated the importance of the C-terminal half of the sigma-1 receptor for ligand binding.[26,28,31] For example, Ganapathy et al.[32] has shown that a sigma-1 receptor splice variant in Jurkat human T lymphocyte cells lacking amino acids 119−149 had a reduced level of [^{3}H]haloperidol binding. Specifically, substitutions of aspartate 126 and glutamate 172 with glycine completely abolished binding of [^{3}H]haloperidol to the sigma-1 receptor[31] in partial support for the idea that the C-terminus is important for ligand binding. In expanding our previous work on the binding of *N*-(3-phenylpropyl)alkan-1-amines and *N*-[3-(4-nitrophenyl)propyl]alkan-1-amines (PPC4, PPC7, PPC12, PPC18, 4-NPPC4, 4-NPPC7, 4-NPPC12, and 4-NPPC18)[13] to the sigma-1 receptor, here we report a high-yield covalent photoincorporation of one of these molecules, 4-NPPC12. The resulting photo-cross-linking of 4-NPPC12 to the sigma-1 receptor resulted in an increase in the electrophoretic mobility (by 3 kDa) of half of the population of the sigma-1 receptor molecules. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF-TOF) and tandem mass spectrometry identified histidine 154 as the site of 4-NPPC12 covalent photoincorporation. Mechanistically, the previously proposed "half-site" reactivity of binding of [^{3}H]-(+)-pentazocine to the pure sigma-1 receptor suggests that 4-NPPC12 may bind to a dimer of the receptor with a 2:1 receptor monomer:ligand ratio.

EXPERIMENTAL PROCEDURES

Reagents and Constructs. [^{3}H]-(+)-Pentazocine and [^{3}H]-(+)-1,3-ditolylguanidine (DTG) were obtained from Perkin-Elmer (Waltham, MA). *N*-(3-Phenylpropyl)-*N*-alkylamines and *N*-[3-(4-nitrophenyl)propyl]-*N*-alkylamines were synthesized previously.[13] The guinea pig maltose binding protein−sigma-1 receptor fusion protein was created previously.[33] All sigma-1 receptor mutants, including the histidine 154 alanine (H154A), cysteine 94 alanine (C94A), and cysteine 94 alanine/methionine 170 cysteine (C94A/M170C) mutants, were created in the pcDNA3.1 plasmid using site-directed mutagenesis (Invitrogen, Carlsbad, CA). The anti-sigma-1 receptor antibody was developed and characterized as described previously,[33] while the sequence specific antibodies against amino acids 52−69 or 143−165[2] were donated by T. Hayashi and T. P. Su (National Institute on Drug Abuse, Bethesda, MD).

Expression and Purification of the Sigma-1 Receptor from *Escherichia coli*. The procedure for the purification of the guinea pig sigma-1 receptor was described previously.[33] Briefly, *E. coli* strain BL21(DE3) (Novagen, Madison, WI) containing the maltose binding protein−sigma-1 receptor six-histidine fusion protein was grown to an OD_{600} of 0.6 before being induced with 0.5 mM IPTG for 4 h at 37 °C. Cells were collected by centrifugation, and the *E. coli* pellet was resuspended in buffer I [20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM 2-mercaptoethanol, and 1 mM EDTA]. The cell suspension was sonicated using a Branson soniWer 250 employing a 1 cm probe (50% output, 2 s bursts, 1 s lag) for 15 min on ice. The cell lysate was centrifuged at 100000*g* for 1 h to separate total particulate and soluble proteins. The particulate fraction was extracted with Triton X-100 (TX-100) at a 4:1 detergent:total protein ratio (w/w) for 3 h while being gently stirred at 4 °C. The extracted material was centrifuged again at 100000*g* for 1 h, and the supernatant was diluted with buffer I to obtain a Triton X-100 concentration of 1%. Proteins were loaded onto an amylose column (New England Biolabs, Ipswich, MA) and washed once with 5 column volumes of buffer II [20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM 2-mercaptoethanol, 1 mM EDTA, and 0.5% TX-100] and once with 3 column volumes of buffer III [20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2$, and 0.5% TX-100]. The MBP−sigma-1 receptor fusion protein was eluted with 3 column volumes of buffer IV [20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2$, 10 mM maltose, and 0.5% TX-100].

The pure MBP−sigma-1 receptor fusion protein was cleaved with Factor Xa protease (Novagen) in 5 mL fractions at room temperature for 24−48 h and the cleavage monitored by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The sigma-1 receptor from the Factor Xa cleavage was purified with HIS-Select HC Nickel affinity gels (Sigma, St. Louis, MO) in batch format. A slurry of proteins and Ni^{2+} beads was tumbled overnight at 4 °C, then washed three times with buffer V [50 mM Na_2HPO_4 (pH 8), 200 mM NaCl, and 0.5% TX-100], and eluted with buffer VI [50 mM Na_2HPO_4 (pH 8), 200 mM NaCl, 250 mM imidazole, and 0.5% TX-100] at room temperature.

Preparation of Guinea Pig Liver Membranes (GPLM) and Rat Liver Membranes (RLM). Membranes were prepared as described previously[29] from frozen tissues (Pel Freez Biologicals, Rogers, AR). The liver tissue was homogenized (10 mL of buffer/g of wet tissue) by four bursts of 10 s each using a brinkman polytron (American Laboratory Trading Inc., East Lyme, CT) on setting 6 in ice-cold sodium phosphate buffer (10 mM, pH 7.4) containing 0.32 M sucrose and a cocktail of protease inhibitors [20 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor, 100 μM phenylmethanesulfonyl fluoride (PMSF), 100 μM benzamidine, and 1 mM EDTA]. The membrane suspension after homogenization was centrifuged for 10 min at 17000*g*, and the supernatant was further centrifuged at 100000*g* to collect the membrane fraction. The pellet from the 100000*g* centrifugation was resuspended in the same buffer described above, snap-frozen, and stored at −80 °C and a protein concentration of 10 mg/mL.

Transient Expression of the Sigma-1 Receptor in COS-7 Cells. The guinea pig sigma-1 receptor in pcDNA3.1 was transfected into COS-7 cells by electroporation and grown for 48 h before being harvested with trypsin. Cells were then resuspended in 1.5 mL of 1× PBS containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and homogenized by being passed through a 27-gauge syringe 25 times. Protein

concentrations were determined by the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA).

Photolabeling and Western Analyses. Fifty micrograms of guinea pig liver membranes (GPLM) or lysates from COS-7 cells overexpressing the sigma-1 receptor were incubated with the test compounds at 10 μM for 30 min at room temperature. The reaction mixtures were then illuminated for 10 s with a high-pressure AH6 mercury lamp to activate the photoprobe followed by separation on a 12% SDS−polyacrylamide gel. Proteins were transferred to a polyvinyldifluoride (PVDF) membrane (Millipore, 0.45 μm) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (pH 10.5) containing 0.5% (w/v) DTT and 15% (v/v) methanol at 65 V for 1 h at 4 °C. The PVDF membrane was blocked with 5% nonfat dry milk for 1 h at room temperature, probed with the anti-sigma-1 receptor antibody overnight at 4 °C, and washed three times for 10 min each in PBS followed by incubation with secondary anti-rabbit IgG HRP for 1 h at room temperature. The membrane was then washed thoroughly and developed with enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL). Protection experiments were performed by pretreating the protein samples with various sigma-1 receptor ligands prior to the addition of 4-NPPC12. Western analyses of the sigma-1 receptor with sequence specific antibodies were performed similarly.

Endoproteinase LysC (EndoLysC) Digestion. EndoLysC cleavage of the photolabeled sigma-1 receptor was performed according to the method of Pal et al.[27] The *E. coli*-expressed sigma-1 receptor was first labeled with 4-NPPC12 followed by separation on a 12% SDS gel. The S1R26 kDa and the S1R23 kDa bands were separately excised from the gel, macerated, and eluted with 1 mL of water by being tumbled overnight at room temperature. The supernatant containing the sigma-1 receptor was collected and concentrated to 100 μL by lyophilization and treated with EndoLysC (0.25 μg) overnight at room temperature. The digestion was terminated by adding 5× SDS−PAGE sample buffer, and samples were resolved on a 16.5% SDS−Tricine/PAGE gel. The protein samples were transferred to a PVDF membrane for Western analyses as described above using the sequence specific antibodies against amino acids 52−69 or 143−165, the polyclonal sigma-1 receptor developed in our laboratory,[33] or the C-terminal hexahistidine tag antibody (Invitrogen).

"In Gel" Digestion with Trypsin and Mass Spectrometry Determination. In gel digestion and mass spectrometric analyses were performed at the Mass Spectrometry Facility (Biotechnology Center, University of Wisconsin—Madison, Madison, WI). For detailed methodologies, see the Supporting Information.

Saturation Binding of [^{3}H]-(+)-Pentazocine and [^{3}H]DTG. Saturation binding of [^{3}H]-(+)-pentazocine to the wild-type (WT) sigma-1 receptor and the histidine 154 alanine mutant (H154A) or [^{3}H]DTG to the cysteine 94 alanine (C94A) and cysteine 94 alanine/methionine 170 cysteine (C94A/M170C) mutants was conducted in a total volume of 100 μL containing 30 μg of the total cell lysates from COS-7 cells overexpressing each mutant in 50 mM Tris-HCl (pH 8) and concentrations of [^{3}H]-(+)-pentazocine from 1 to 100 nM for 60 min at 30 °C. The reaction was terminated by rapid filtration through glass fiber filters (Whatman GF/B, Whatman, Maidstone, U.K.), using a Brandel cell harvester (Brandel, Gaithersburg, MD). The glass fiber filters were presoaked in 0.5% polyethyleneimine (PEI) for at least 1 h at room temperature. Filters were washed four times with 4 mL of ice-cold 50 mM Tris-HCl (pH 8.0). Radioactivity was quantified by liquid scintillation (Ultima Gold, Perkin-Elmer) counting using a liquid scintillation counter (Packard model 1600CA, Packard Instrument Co., Downers Grove, IL).

Competitive Displacement of [^{3}H]-(+)-Pentazocine Binding. The conditions for the competition displacement of [^{3}H]-(+)-pentazocine to the WT and H154A sigma-1 receptors were similar to those for saturation binding except all samples contained a final [^{3}H]-(+)-pentazocine concentration of 10 nM and increasing concentrations of 4-NPPC12 between 3 nM and 100 μM. IC_{50} values were then converted to K_I values using the Cheng−Prusoff correction[34] with the equation $K_I = IC_{50}/(1 + [L]/K_D)$, where [L] is the ligand concentration and K_D is the previously determined dissociation constant for [^{3}H]-(+)-pentazocine (10 nM).

RESULTS

Previously, we reported the synthesis and evaluation of *N*-(3-phenylpropyl) and *N*-[3-(4-nitrophenyl)propyl] derivatives of *N*-alkylamine with varying chain lengths (Figure 1A) as high-affinity ligands for sigma-1 and sigma-2 receptors.[13] Binding studies showed that affinities increased linearly with carbon chain length and maximal binding was achieved with 12 carbons. Additionally, four of these *N*-alkylamine derivatives (PPC7, 4-NPPC7, PPC12, and 4-NPPC12) showed high selectivity for the sigma-1 and sigma-2 receptors when assessed in a binding screen of more than 40 other membrane receptor/transporter targets, and these four compounds have demonstrated promising anticancer activities against an array of cancer cell lines. At present, we report the discovery of photo-cross-linking by one of these molecules, 4-NPPC12, to the sigma-1 receptor. The unique photochemistry of 4-NPPC12 allowed its covalent photoincorporation into the sigma-1 receptor and resulted in an alteration of the electrophoretic migration pattern of the receptor on SDS gels. Illumination using a high-energy mercury lamp ($h\nu$) in the presence of an equimolar ratio of the pure sigma-1 receptor and 4-NPPC12 produced a secondary species, the S1R23 kDa form (Figure 1B). The secondary species was named for its apparent molecular mass of 23 kDa on SDS−polyacrylamide gels, approximately 3 kDa smaller than the sigma-1 receptor apparent gel size of 26 kDa (Figure 1B). Furthermore, we found that while light alone produced less than 10% of the S1R23 kDa species (Figure 1B, lane 2), 4-NPPC12 in combination with light maximized the production of the S1R23 kDa form up to 50% of the total protein used in each reaction (Figure 1B, lane 4). We could also detect the S1R23 kDa species in both COS-7 cells overexpressing the guinea pig sigma-1 receptor and in guinea pig liver membranes (Figure 1B, middle and bottom) using a full-length sigma-1 receptor polyclonal antibody.[33] The generation of the S1R23 kDa form from both membrane preparations confirmed that the pure sigma-1 receptor is structurally similar to the receptor in situ, justifying our use of the pure sigma-1 receptor in subsequent studies. Because the gel migration pattern of the S1R23 kDa species could have been due to a nonspecific photochemical reaction of 4-NPPC12 with the sigma-1 receptor, we found that preincubation with a putative sigma-1 receptor antagonist, haloperidol and DTG, could block the formation of the S1R23 kDa form (Figure 1B, lane 5, and Figure S2C of the Supporting Information), indicating specific photo-cross-linking at (or near) the haloperidol binding site. Moreover,

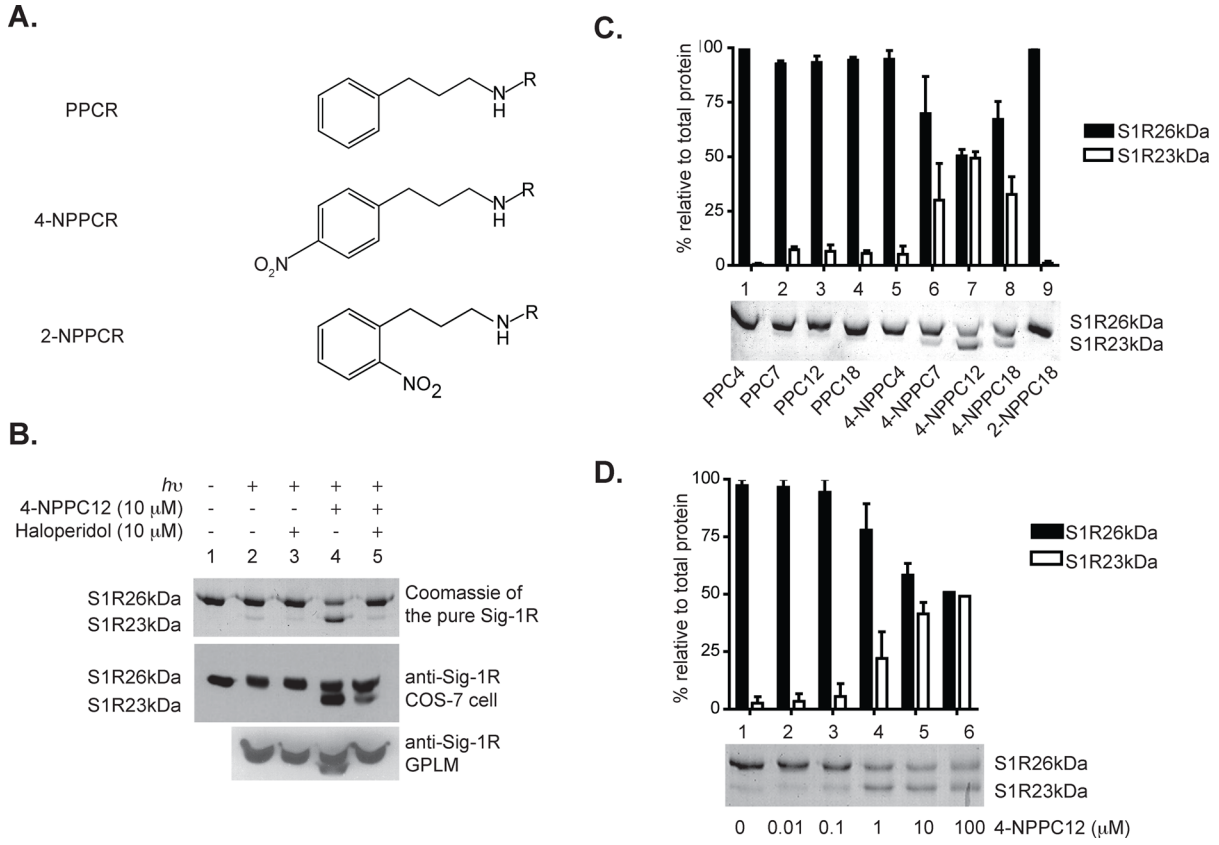


Figure 1. Asymmetric photo-cross-linking of 4-NPPC12 to the sigma-1 receptor. (A) Chemical structures of *N*-alkylamine derivatives: PPC4 and 4-NPPC4, where R = *n*-butyl; PPC7 and 4-NPPC7, where R = *n*-heptyl; PPC12 and 4-NPPC12, where R = *n*-dodecyl; PPC18, 4-NPPC18, and 2-NPPC18, where R = *n*-octadecyl. These compounds were previously reported.[13] (B) Light- and 4-NPPC12-dependent formation of the 23 kDa sigma-1 receptor observed in different preparations of the sigma-1 receptor: Coomassie-stained gel of the pure sigma-1 receptor (top), lysates from COS-7 cells overexpressing the sigma-1 receptor, detected via Western blots (middle), and guinea pig liver microsomes (GPLM), also detected via Western blotting (bottom). (C) Formation of the lower 23 kDa species was determined to be dependent on alkyl chain length and the presence of a 4-nitro moiety. A representative Coomassie-stained gel of the pure sigma-1 receptor treated with varying chain length *N*-alkylamine derivatives in the presence of light is shown. (D) The 23 kDa sigma-1 receptor was also dependent on the concentration of compound 4-NPPC12, as shown from Coomassie staining of the pure sigma-1 receptor. Each experiment was performed three times (the error bar is the standard error of the mean).

we were unable to detect the S1R23 kDa species when the pure protein was predenatured with 1% SDS (data not shown).

We next explored the structural requirements of *N*-alkylamine derivatives leading to the formation of the S1R23 kDa species. Because a number of *N*-alkylamine derivatives were available (Figure 1A),[13] we first determined the contribution of the nitro moiety and/or carbon chain length of *N*-alkylamine derivatives to the generation of the S1R23 kDa species. Non-nitro (PPC4, PPC7, PPC12, and PPC18) and 2-nitro (2-NPPC18, K_I value of 76 ± 3 nM at the pure sigma-1 receptor) molecules failed to produce the S1R23 kDa species (Figure 1C, lanes 1−4), while three of the 4-nitro molecules (4-NPPC7, 4-NPPC12, and 4-NPPC18) generated the S1R23 kDa form (Figure 1C, lanes 6−8). Interestingly, the *N*-butylamine nitro derivative, 4-NPPC4, was also unable to produce the S1R23 kDa species (Figure 1C, lane 5), but compounds with at least seven carbons all produced this secondary sigma-1 receptor form. While the mobility shift pattern of the S1R23 kDa form suggested an affinity dependency, 4-NPPC7, with an affinity of 7.5 nM, was still less efficient than 4-NPPC12 (K_I value of 32 nM) at producing the secondary species. These results indicated that the photo-cross-linking of 4-NPPC12 to the sigma-1 receptor is carbon chain length-dependent and that the dodecylamine nitro derivative, 4-NPPC12, is optimally situated to react with the sigma-1 receptor within the binding pocket. Next, we examined the dependence of ligand concentration for the formation of the secondary S1R23 kDa species. First, we found that treatment with up to 100 μM 4-NPPC12, 10-fold above the receptor concentration, did not enhance the production of the S1R23 kDa form beyond 50% (Figure 1D). Second, the formation of the S1R23 kDa species was independent of the time of light exposure because multiples of 10 s light bursts and extended light exposure up to 60 s (data not shown) did not enhance the formation of the S1R23 kDa species. The generation of the S1R23 kDa form was obtained maximally at an appropriate 1:1 molar ratio of the S1R26 kDa and S1R23 kDa forms in these experiments.

In an attempt to characterize the differences between the S1R26 kDa and S1R23 kDa forms, we took two approaches: (1) EndoLysC cleavage of both forms of the receptors followed by detection of the peptide fragments with sequence specific antibodies and (2) MALDI-TOF-TOF analysis of peptides generated from trypsin digestion of both forms. Figure 2A shows the linear representation of the sigma-1 receptor and its EndoLysC-generated peptides (17 and 9.8 kDa). Western analyses using a C-terminal hexahistidine antibody indicated that the 9.8 kDa peptide produced from the S1R23 kDa species migrated lower than that of the 9.8 kDa equivalent from the S1R26 kDa species (Figure 2B, second western). Furthermore,

dx.doi.org/10.1021/bi301517u | *Biochemistry* 2013, 52, 859−868

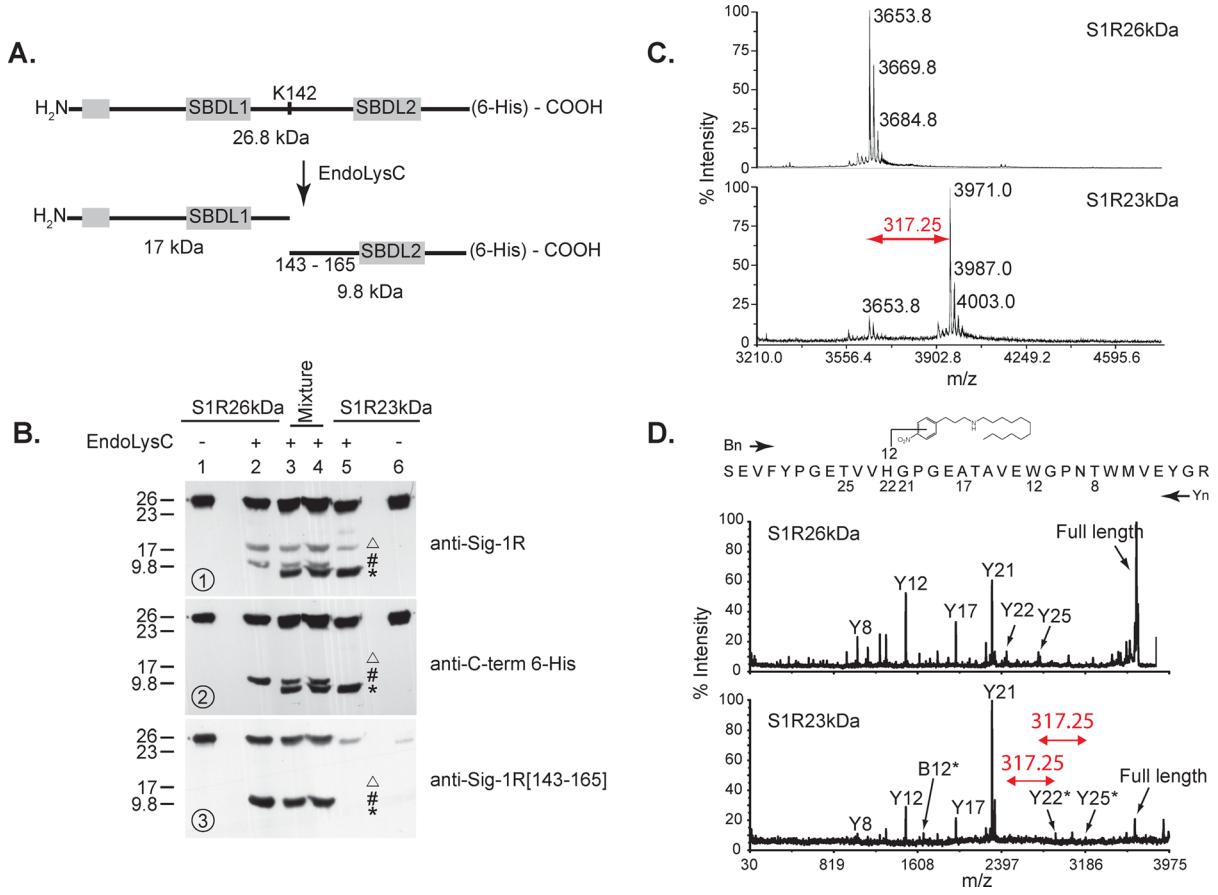


Figure 2. Identification of the 4-NPPC12 binding region on the pure sigma-1 receptor. (A) Linear sequence representation of the guinea pig sigma-1 receptor and its EndoLysC cleavage patterns. SBDL1, amino acids 91−109; SBDL2, amino acids 176−194. (B) Gel mobility shift of the lower 23 kDa species mapped to the C-terminal region of the sigma-1 receptor. Shown are Western blots of peptides generated from the EndoLysC cleavage of the S1R26 kDa and S1R23 kDa forms with various sequence specific antibodies. Antibody against the full-length sigma-1 receptor (Sig-1R Ab), sequence specific antibodies against amino acids 143−165 ([143−165]Sig 1R Ab), and antibodies against the C-terminal six-histidine tag (C-term 6-His Ab): 17 kDa (△), 9.8 kDa from the S1R26 kDa species (#), and 9.8 kDa peptide from the S1R23 kDa species (∗). (C) MALDI-TOF-TOF analyses localized the 4-NPPC12 derivative to the peptide region of amino acids 143−175. Shown are selected spectral regions of both S1R26 kDa and S1R23 kDa forms. 4-NPPC12 photolysis resulted in the addition of 317.25 Da to the tryptic peptide corresponding to amino acids 143−175 generated from the S1R23 kDa form (bottom) but not the S1R26 kDa form (top). For the complete MALDI-TOF-TOF results, see Figure S1 of the Supporting Information. (D) MS/MS mapping identified histidine 154 as the site of 4-NPPC12 photo-cross-linking to the lower S1R23 kDa form of the sigma-1 receptor. Shown are MS/MS spectra of the peptides of amino acids 143−175 from both the underivatized S1R26 kDa (top) and the derivatized S1R23 kDa (bottom) forms. Yn designates peptides generated from the C-terminus toward the N-terminus, and Bn designates peptides generated from the N-terminus toward the C-terminus of the parent tryptic peptide of amino acids 143−175, where *n* is the amino acid position in either direction. Asterisks denote peptides that were shifted by 317.25 Da.

we found that while the sequence specific antibody against amino acids 143−165 recognized the 9.8 kDa peptide from the S1R26 kDa form (Figure 2B, third western, lanes 2−4), this same antibody failed to detect the 9.8 kDa peptide generated from the S1R23 kDa species (Figure 2B, third western, lanes 3−5). The discovery that the sequence specific antibody for amino acids 143−165 failed to detect its epitope on the S1R23 kDa species suggested that 4-NPPC12 covalently derivatized the sigma-1 receptor in this region.

We therefore used MALDI-TOF-TOF analysis as a second approach to deduce the region of 4-NPPC12 photoincorporated into the S1R23 kDa form. Trypsin protease was chosen because the guinea pig sigma-1 receptor contains two lysines and several arginines and tryptic peptides generated from both the S1R26 kDa and S1R23 kDa forms would also be within the detection limits of the MALDI-TOF-TOF analyses. While MALDI-TOF-TOF provided an overall sequence coverage of 55%, the C-terminal region of interest, amino acid residues 120−223, which were predicted to contain the reaction product of 4-NPPC12, was almost completely sequence covered (see Figure S1 and Tables S1 and S2 of the Supporting Information for detailed results from MALDI-TOF-TOF experiments). MALDI-TOF-TOF spectral regions between 3210 and 4594 Da of the S1R26 kDa and S1R23 kDa species were selected to highlight the differences between these two forms of the sigma-1 receptor created by the reaction with 4-NPPC12 (Figure 2C). For complete MALDI-TOF-TOF spectra of both the S1R26 kDa and S1R23 kDa species, see Figure S1 of the Supporting Information. A tryptic peptide corresponding to amino acids 143−175 from the S1R26 kDa species produced a molecular mass of 3653.8 Da (3669.8 Da represents the single-methionine-oxidized form and 3684.8 Da the double-methionine-oxidized form) closely matched the predicted size. However, the same peptide from the S1R23 kDa species produced a molecular mass of 3971.0 Da (and 3987.0 Da represents the single-methionine-oxidized form and 4003.0 Da the double-methionine-oxidized

dx.doi.org/10.1021/bi301517u | *Biochemistry* 2013, 52, 859−868

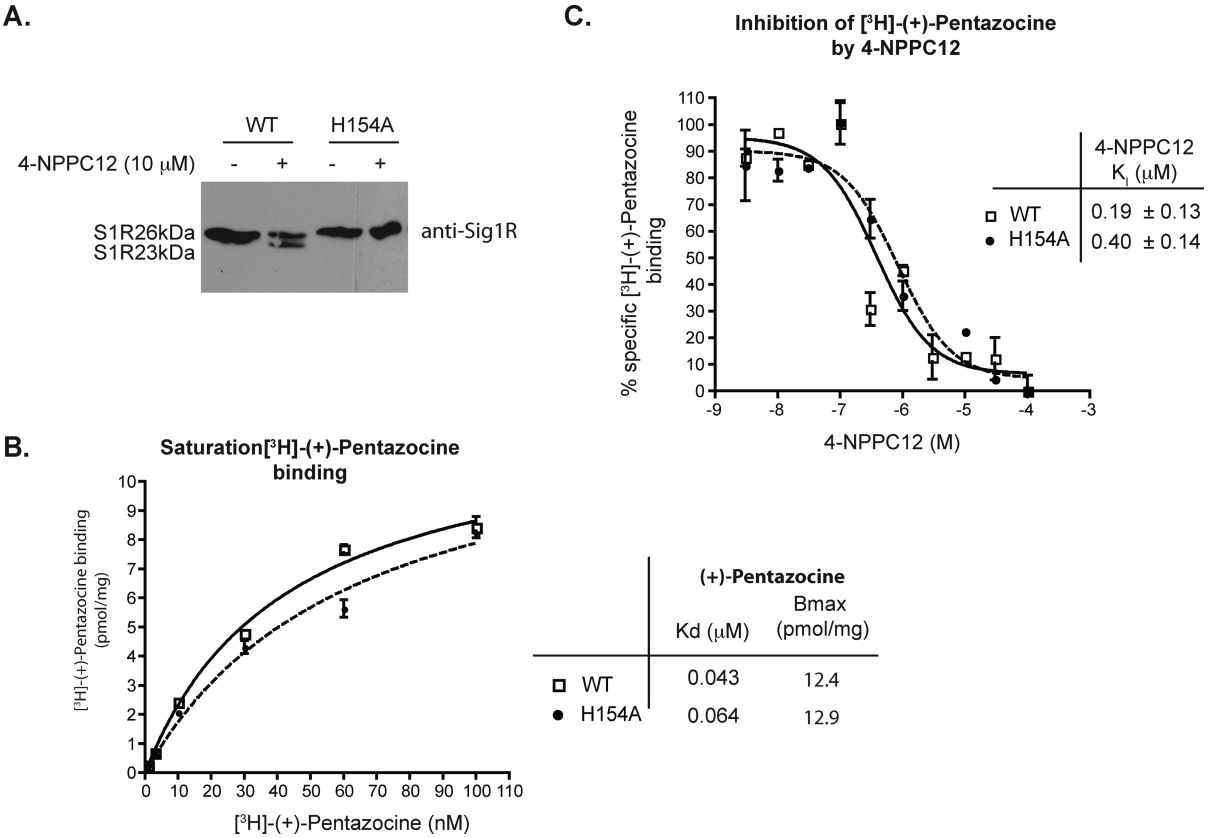


Figure 3. H154A mutant that binds equally efficiently to [^{3}H]-(+)-pentazocine and 4-NPPC12 but failed to form the S1R23 kDa species upon photolysis. (A) Mutation of histidine 154 to alanine of the guinea pig sigma-1 receptor resulted in a failure of this mutant to form the lower 23 kDa species in a light- and 4-NPPC12-dependent manner. Shown is a Western blot of cell lysates from COS-7 cells transfected with either the WT or H154A mutant and photolyzed with 4-NPPC12. (B) The sigma-1 receptor H154A mutant retains binding to pentazocine. Saturation binding of [^{3}H]-(+)-pentazocine with lysates of COS-7 cells transfected with either the WT or the H154A mutant. (C) The sigma-1 receptor H154A mutant binds to 4-NPPC12 like the WT receptor. Inhibition of [^{3}H]-(+)-pentazocine binding to determine the K_I values for 4-NPPC12 for the WT sigma-1 receptors and the H154A mutant. All binding experiments were performed three times each in triplicate (the error bar is the standard error of the mean of the average).

form), which is 317.25 Da higher than the mass of its equivalent peptide generated from trypsin digestion of the S1R26 kDa form.

MS/MS of the modified peptide of amino acids 143−175 from the S1R23 kDa species showed that amino acid histidine 154 contained a 317.25 Da adduct (Figure 2D and Tables S3 and S4 of the Supporting Information). As summarized in Figure 2D (and Tables S3 and S4 of the Supporting Information), while the observed mass of peptide Y21 (GPGEATAVEWGPNTWMVEYGR) matched its theoretical mass, Y22 (**H**GPGEATAVEWGPNTWMVEYGR), which contains an additional histidine, showed an increase of 317.25 Da. Likewise, peptide B12 (SEVFYPGETVV**H**) was also modified by an addition of 317.25 Da. The tryptic peptide of amino acids 143−175 from the S1R26 kDa species was treated similarly and showed the absence of the 317.25 Da addition (Figure 2D and Table S3 of the Supporting Information), which validated the MALDI-TOF-TOF results. Taken together, these results indicated that 4-NPPC12 covalently modified the S1R23 kDa form at histidine 154. While the mass of 317.25 Da was 31.1 Da smaller than the molecular mass of 4-NPPC12 (348.25 Da), the adduct would be consistent with a loss of NOH from the phenyl ring region.

To confirm that histidine 154 was the site of 4-NPPC12 photoincorporation, we substituted histidine 154 with alanine by site-directed mutagenesis. As shown in Figure 3A, while photolysis with 4-NPPC12 produced the S1R23 kDa species in membranes of COS-7 cells overexpressing the WT sigma-1 receptor, the removal of histidine 154 abrogated the ability of this compound to produce the S1R23 kDa form. Importantly, the failure of 4-NPPC12 to produce the S1R23 kDa form was not due to an alteration of the ligand binding affinity because both [^{3}H]-(+)-pentazocine (Figure 3B) and specifically 4-NPPC12 (Figure 3C) bound equally well to the H154A mutant and the WT receptor. These results indicate that histidine 154 is situated in or close to the ligand binding site to allow a photochemical reaction with 4-NPPC12 to occur but does not necessarily participate directly in the binding of ligand. Together, these data supported the conclusion that histidine 154 is the site of photoderivatization by 4-NPPC12.

Finally, it is important to note that the 317.2 Da adduct formed at histidine 154 is 31 Da smaller than the molecular mass of 4-NPPC12 (348.2 Da). Possibilities to account for the loss of 31 Da include (1) a loss of two oxygens with the addition of a proton and (2) the loss of a nitrogen, an oxygen, and a proton. A possible mechanism for covalent modification of histidine 154 by 4-NPPC12 may involve a photochemical rearrangement of 4-NPPC12 at the α-carbon of the alkyl side chain to produce a double-bond Michael acceptor. This would promote reaction with one of the amino groups of the imidazole ring (2-NPPC12, on the other hand, is likely to photochemically rearrange so that the proximal nitro group reacts

dx.doi.org/10.1021/bi301517u | *Biochemistry* 2013, 52, 859−868

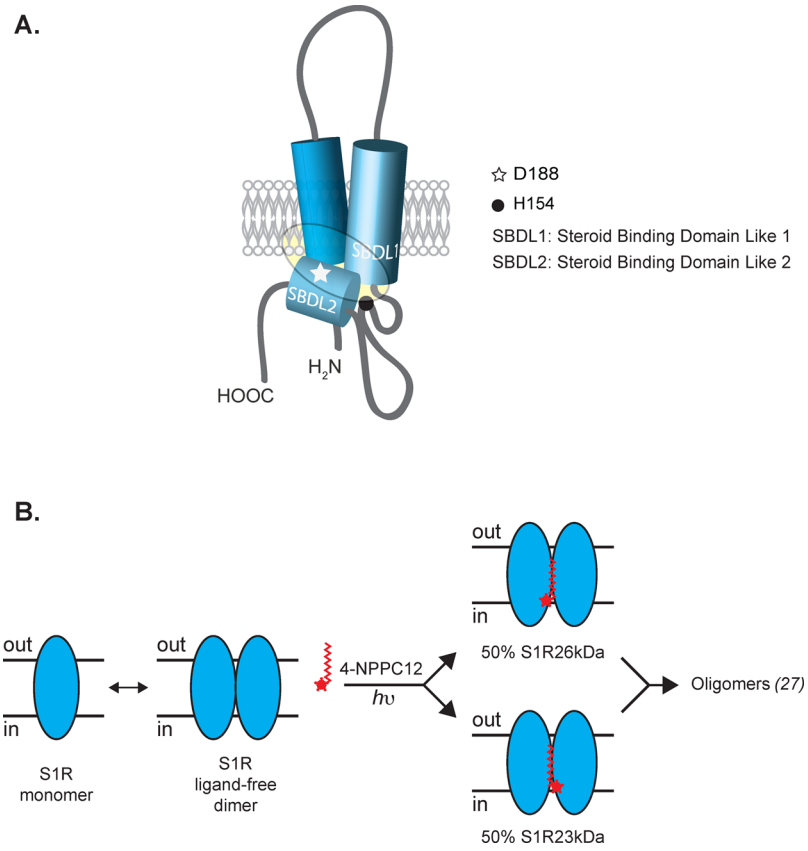


Figure 4. Models of the sigma-1 receptor ligand binding region. (A) Model of the sigma-1 receptor binding region from previous photolabeling studies and from the results presented here with the derivatization by 4-NPPC12. D188, aspartate 188; H154, histidine 154. The shaded area represents the ligand binding region. (B) Proposed model of the sigma-1 receptor dimer in the presence of 4-NPPC12. The sigma-1 receptor may exist naturally in equilibrium among monomeric, dimeric, and/or oligomeric forms.

intramolecularly, thus preventing a reaction of the imidazole ring histidine 154). Detailed characterization of the mechanism of the light-dependent reaction between histidine and 4-NPPC12 is beyond the scope of this work, but future studies aiming at the elucidation of the photochemistry of the reaction of 4-nitrophenylpropyl compounds with imidazoles are warranted.

DISCUSSION

Interest in the sigma-1 receptor has mainly revolved around its promiscuity in ligand binding to a wide range of pharmacologically important molecules. Determination of the ligand binding site therefore should assist in future designs of more selective ligands targeting the sigma-1 receptor as potential therapeutics against cancer, neurodegeneration, and psychosis. Previously, we synthesized and evaluated the binding characteristics of varying chain length *N*-alkylamines and their derivatives for the sigma-1 receptor.[13] In studying the ligand binding region of *N*-alkylamine derivatives for the sigma-1 receptor, we uncovered a unique photodependent property of three *N*-[3-(4-nitrophenylpropyl)]alkan-1-amines (4-NPPC7, 4-NPPC12, and 4-NPPC18). Photolysis of 4-NPPC7, 4-NPPC12, or 4-NPPC18 with the sigma-1 receptor produced a novel secondary sigma-1 receptor form that migrated at 23 kDa (Figure 1A), approximately 3 kDa below its apparent migration patterns on SDS gels. From MALDI-TOF-TOF and MS/MS, 4-NPPC12 was found to be covalently derivatized to the S1R23 kDa form at histidine 154 (Figure 2C,D), which partly explains the gel mobility shift of the full-length S1R23 kDa form and its EndoLysC-generated 9.8 kDa peptide (Figure 2B). Anomalous SDS−PAGE migration of some membrane proteins has been demonstrated to result from altered SDS binding related to the secondary structures of the proteins.[35] Interestingly, the conjugation of a rat microtubule-associated light chain 3 (LC3) by phosphatidylethanolamine (PE), a marker of autophagy, also showed a downward shift in gel mobility of 3 kDa.[36] While it is generally believed that an increase in hydrophobicity (i.e., PE conjugation to LC3 or the derivatization of 4-NPPC12 to the S1R23 kDa species) allowed more SDS loading, thus retarding the electrophoretic mobility of proteins, in both cases the modified proteins migrated faster than their native forms. Hence, it may be a general phenomenon that lipidated forms of some proteins migrate faster on SDS−PAGE by as yet unknown mechanisms.

MS/MS results indicated that 4-NPPC12 formed a 317.25 Da adduct at histidine 154 of the S1R23 kDa species. Substitution of histidine 154 with alanine, while preventing the formation of the S1R23 kDa species induced by 4-NPPC12, did not alter binding of 4-NPPC12 and/or [^{3}H]-(+)-pentazocine (Figure 3). We showed previously that SBDLI, SBDLII, and part of the TM1 region form the binding site of the sigma-1 receptor through the use of a number of photoaffinity labels.[26−28] One of these photoprobes, [^{125}I]IACoc, was found to specifically photoinsert at aspartate 188.[26] The covalent cross-linking of 4-NPPC12 to histidine 154 has prompted new ideas regarding the sigma-1 receptor ligand-binding region. In the model presented in Figure 4A, the loop region between SBDLI and SBDLII is proposed to fold back to create a ligand binding pocket together with previously defined regions (Figure 4A, shaded area). Ganapathy et al.[32] demonstrated that a sigma-1 receptor

dx.doi.org/10.1021/bi301517u | *Biochemistry* 2013, 52, 859−868

splice variant in Jurkat human T lymphocyte cells lacking amino acids 119−149 was unable to bind to [^{3}H]haloperidol. We expressed this splice variant in COS-7 cells and found that photo-cross-linking with 4-NPPC12 did not produce the S1R23 kDa form (data not shown), indicating the structural importance of this region for binding of the ligand to the sigma-1 receptor. Mutations of aspartate and glutamate 172 to glycine completely abolished [^{3}H]haloperidol binding,[31] suggesting that the linker sequence between SBDLI and SBDLII is also important for ligand binding.

Another important observation from this work was the stoichiometric generation of the S1R23 kDa form with 4-NPPC12. The light-dependent 4-NPPC12-induced formation of the S1R23 kDa form occurred maximally at a 1:1 S1R26 kDa form:S1R23 kDa form ratio. While the precise mechanism leading to this observation is unclear, we favor a hypothesis whereby the sigma-1 receptor forms a homodimer or an oligomer containing dimers in which 4-NPPC12 binds at the interface of the homodimer yet only photo-cross-linked stoichiometrically to one subunit of the dimer (Figure 4B). Multiple lines of evidence from our lab have suggested that the sigma-1 receptor may exist as a dimer or an oligomer in native membranes and in a detergent solution. First, Pal et al.[27] found that both [^{125}I]IACoc and [^{125}I]IAF labeled oligomeric forms of sigma-1 receptors in rat and guinea pig liver membranes. Second, Ramachandran et al.[33] reported that the sigma-1 receptor expressed and purified from *E. coli* exhibited a 1:2 [^{3}H]-(+)-pentazocine:sigma-1 receptor molar ratio. Third, in COS-7 cells overexpressing the myc- and HA epitope-tagged sigma-1 receptor, co-immunoprecipitation using either one of the epitopes resulted in the pull down of the other epitope-tagged sigma-1 receptor as evidenced by Western blotting (unpublished data). Finally, we have found here that upon mutating methionine 170 to cysteine in a receptor with a cysteine-less background, the M170C mutant formed a SDS-resistant disulfide-linked dimer (as assessed by Western blotting using an anti-sigma-1 receptor antibody) that was eliminated by a reducing agent (Figure S2A of the Supporting Information). The sigma-1 receptor M170C mutant was found to possess a binding affinity similar to that of the WT as assessed by saturating binding of [^{3}H]DTG (Figure S2B of the Supporting Information). When considered collectively, our interpretation is that the sigma-1 receptor can naturally form dimers and/or oligomers. Together, these data evoke a model whereby the pure sigma-1 receptor exists as a homodimer and/or an oligomer containing dimers and binds to ligand with a ratio of one ligand (in this case 4-NPPC12) per dimer. We propose the following model to explain these results. 4-NPPC12 binds to the interface between two monomer subunits but reacts once with equal efficiency with either subunit, which produces a 1:1 ratio of the unmodified form (S1R26 kDa) to the modified form (S1R23 kDa) of the sigma-1 receptor (Figure 4B). Alternatively, a second possibility could exist in which the sigma-1 receptor forms a structurally asymmetric homodimer and 4-NPPC12 preferentially reacts with only one subunit. A recent report about the structure of the abscisic acid (ABA) receptor (PYR1) in *Arabidopsis thaliana* showed that PYR1 forms an asymmetric homodimer in which only one monomer binds to ABA.[37] The asymmetry of PYR1 is created by a *cis*−*trans* isomerization of a proline (Pro88) on the ligand-bound monomer such that ABA is trapped in the ligand binding cavity. The isomerization also induced a conformational change in the protein domain at the interface of the PYR1 homodimer, allowing allosteric modulation by the isomerized subunit. Therefore, it is possible that the histidine 154 region is located at a dimerization interface and mediates conformational changes between homodimer subunits of the sigma-1 receptor during ligand binding. Additionally, the proposed interaction of 4-NPPC12 with the receptor may also be explained by the formation of higher-order oligomers of the sigma-1 receptor with a dimer as the smallest functional unit (Figure 4B). Evidence of sigma-1 receptor dimerization is also supported by the protein sequence. We found that the sigma-1 receptor contains two GXXXG putative oligomerization motifs: the first one located at TM2 (amino acids 87−91) and the second in the region flanking the C-terminus of SBLDII (amino acids 108−112). These sequences have been shown to promote the formation of dimers in membrane-bound glycophorin A[38] and oligomers in the membrane-bound ABC transporter, ABCG2.[39] It may be possible that the first GXXXG sequence mediates the dimerization of the sigma-1 receptor while the second GXXXG sequence drives the formation of oligomers.

While the physiological significance of the sigma-1 receptor dimer in the context of the cellular environment is unclear, a recent report demonstrated that mutation of glutamic acid 102 to glutamine (E102Q) in the sigma-1 receptor produced a detergent-resistant apparent homodimer as assessed by SDS−PAGE.[40] The sigma-1 receptor harboring an E102Q mutation was suggested by the authors to cause juvenile amyotrophic lateral sclerosis (JALS) in a consanguineous family with a high familial incidence of this neurodegenerative disease. Thus, it will be important in the future to determine the functional forms of the sigma-1 receptor in a cellular context and to determine whether different classes of ligands (agonists vs antagonists) vary in their properties in binding to different quaternary forms of the receptor.

■ ASSOCIATED CONTENT

Supporting Information

MALDI-TOF-TOF spectra from analyses of trypsin-digested S1R26 kDa and S1R23 kDa species (Figure S1), evidence of sigma-1 receptor dimer formation (Figure S2), peptide mapping of the S1R26 kDa form after trypsin digestion (Table S1), peptide mapping of the S1R23 kDa form after trypsin digestion (Table S2), MS/MS sequencing of the peptide of amino acids 151−183 from the S1R26 kDa form (Table S3), and MS/MS sequencing of the peptide of amino acids 151−183 from the S1R23 kDa form (Table S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This research was supported by National Institutes of Health Grants MH065503 and GM-33138 (to A.E.R.), the Retina Research Foundation Edwin and Dorothy Gamewell Professorship (to A.E.R.), the University of Wisconsin-Madison Advanced Opportunity Fellowships (AOF), and the Gates Millennium Scholarship (to U.B.C.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Teruo Hayashi and Dr. Tsung Ping Su (National Institute on Drug Abuse) for providing the sequence specific sigma-1 receptor antibodies, Dr. Michael Palte (Department of Biochemistry, University of Wisconsin—Madison) for insightful discussions about the possible photochemistry of 4-NPPC12 and 2-NPPC12, and Dr. Lian Wang Guo for reading the manuscript.

■ ABBREVIATIONS

DMT, *N*,*N*-dimethyltryptamine; DTG, ditolylguanidine; ER, endoplasmic reticulum; GPLM, guinea pig liver membranes; IP_3R-3, inositol trisphophate receptor type 3; MBP, maltose binding protein; SBDL, steroid-binding domain liked; SKF-10,047, *N*-allyl-normetazocine; TM, transmembrane; RLM, rat liver membranes.

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